American Journal of Hematology 61:34–39 (1999)

Activation of Coagulation Factor VII by Tissue-Type Plasminogen Activator

Hiroshi Tsujioka, Akira Suehiro,* and Eizo Kakishita

Second Department of Internal Medicine, Hyogo College of Medicine, 1-1 Mukogawa-cho Nishinomiya, Hyogo 663-8501, Japan.

To investigate the effect of tissue-type plasminogen activator (t-PA) on blood coagulation, we examined the effects of the addition of t-PA to normal pool plasma (NPP) on clotting times such as diluted prothrombin time (PT) and kaolin clotting time (KCT). The diluted PT but not the KCT was significantly shortened by the addition of t-PA to NPP compared with the normal controls, suggesting a t-PA–induced activation of blood coagulation through factor VII (FVII) activation. The activated factor VII (FVIIa) concentration in the NPP was significantly increased by the addition of t-PA. Although the FVIIa formation was not observed following the incubation of purified FVII with only t-PA or plasminogen, an increase in the FVIIa level was observed after the incubation of purified FVII with t-PA together with plasminogen, or only plasmin. This plasmin-mediated FVIIa formation was also confirmed by Western blotting. These findings suggest that t-PA enhances the activation of the coagulation system through FVII activation. Am. J. Hematol. 61:34–39, 1999. © 1999 Wiley-Liss, Inc.

Key words: tissue-type plasminogen activator; activated factor VII; clotting time

INTRODUCTION

Tissue-type plasminogen activator (t-PA) is a trypsin-like serine proteinase that activates plasminogen to plasmin, thereby initiating fibrinolysis. Amounts of t-PA sufficient for clinical investigation recently have been produced in melanoma cell cultures and by recombinant DNA techniques [1]. Thrombolytic therapy using t-PA has been developed in recent years for treating coronary thrombosis in patients with acute myocardial infarction (AMI) [2-6]. However, the effectiveness of t-PA is limited by several problems, including failure of thrombolysis, reocclusion after thrombolysis, and hemorrhage complications.

We previously examined the effect of a plasminogen activator on platelet aggregation to determine whether thrombolytic agents activate platelet function as one of the mechanisms of reocclusion [7,8]. However, we found no significant effect of thrombolytic agents on the platelets. Reocclusion after thrombolysis may be induced by residual thrombus, exposed collagen, or other thrombogenic material at the site of plaque rupture. Reocclusion is usually seen in patients treated without heparin, suggesting the activation of the coagulation system after thrombolytic therapy.

There are several reports concerning the activation of the blood coagulation system after thrombolytic therapy using t-PA. The increased production of thrombin almost immediately after the initiation of a t-PA infusion has been reported based on the rapid increases in the plasma concentrations of fibrinopeptide A (FPA) [9–13], thrombin antithrombin III complex (TAT) [14,15], and prothrombin fragment 1+2 (F1+2) [15,16] documented in clinical studies. These findings suggest that the activation of the blood coagulation system was caused by the intravenous infusion of thrombolytic agents. However, the exact mechanism of the activation of blood coagulation by t-PA has not yet been established.

Although the detailed mechanisms have not yet been well defined, the activation of coagulation factor XII (FXII) by plasmin [17] has been reported to be one of the mechanisms of the activation of coagulation system by t-PA. An increase of coagulation factor FVII (FVII) activity by the addition of plasmin to purified FVII was

*Correspondence to: Akira Suehiro, M.D., Second Department of Internal Medicine, Hyogo College of Medicine, 1-1 Mukogawa-cho Nishinomiya, Hyogo 663-8501, Japan.

Received for publication 13 August 1998; Accepted 6 January 1999

observed using a former method [18], and, in a recent report, FVII activation caused by the addition of t-PA to normal plasma has also been suggested in an investigation using FVII-deficient plasma [19]. In the present study, to identify the detailed mechanism of this FVII activation, we examined the effect of t-PA on the coagulation system by the addition of t-PA in vitro. We observed that the clotting activity increased in vitro in response to the activation of plasminogen with t-PA as a consequence of the plasmin-mediated activation of FVII.

MATERIALS AND METHODS

Chemicals

Recombinant t-PA was obtained from Genentec (San Francisco, CA). Human FVII was obtained from American Diagnostica (Greenwich, CT). Thromboplastin, plasmin, and plasminogen were obtained from Sigma Chemical Co. (St. Louis, MO). Platelin® used as a phosholipid was obtained from Organon Teknika (Durham, NC). A recombinant soluble form of tissue factor protein, which does not promote FVII activation and contains the first 219 amino acids of tissue factor (sTF 1-219) and activated factor VII (FVIIa), were kindly provided by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). Murine monoclonal antibody against human FVII/VIIa was obtained from American Diagnostica. Biotinylated horse antimouse IgG was obtained from Vector Laboratories (Burlingame, CA), as were the VECTASTAIN Elite ABC KIT for a biotinylated horseradish peroxidase conjugate and the AEC (3-amnio-9-ethylcarbazole) substrate kit horseradish peroxidase. Super block™ blocking buffer blotting in TBS was obtained from Pierce Chemical (Rockford, IL). All reagents used were of the highest commercially available grade.

Samples

Normal pool plasma (NPP) was obtained from 12 healthy volunteers. Citrated whole blood (1 volume of 3.8% sodium citrate + 9 volumes of blood) was centrifuged at 3,000 rpm for 15 min, and the supernatant was collected as NPP and stored at −80°C until use.

Clotting Time Assay

The diluted prothrombin time (diluted PT) and kaolin clotting time (KCT) tests were performed in the manual mode [20,21]. An aliquot mixed with 90 μl NPP, 10 μl t-PA and 100 μl of ×500 diluted thromboplastin was incubated for 1, 3, or 5 min at 37°C in a glass tube. Coagulation was then initiated by the addition of 100 μl of 0.025 M $CaCl_2$, and the time until the clot formation confirmed by the naked eye was defined as the diluted PT. An aliquot mixed with 90 μl NPP, 10 μl t-PA and 50 μl of 2% kaolin solution was incubated for 1, 3, or 5 min at 37°C in a glass tube. Coagulation was then initiated by the addition of 100 μl of 0.025 M $CaCl_2$, and the time until the clot formation was defined as the KCT.

FVIIa Assay

The quantitation of the plasma FVIIa levels was performed using sTF 1-219 according to the procedure of Wildgoose et al. [22] in a one-stage clotting assay in the manual mode. Ten percent volume of tPA, plasmin or plasma was added to NPP or purified FVII solution and incubated for 5 min at 37°C. The samples were diluted five-fold in 0.1 M NaCl/0.05 M Tris-HCl/0.1% bovine serum albumin pH 7.4 (TBS/BSA) and mixed with an equal volume of FVII-deficient plasma to yield a total volume of 100 μl. Each aliquot was incubated for 5 min at 37°C with 50 μl of Platelin®. Coagulation was then initiated by the addition of a 100 μl aliquot of 10 ng/ml sTF 1-219 and $CaCl_2$ diluted in 0.1 M NaCl/0.05 M Tris-HCl/1% BSA pH 7.4. The coagulation time was subsequently converted to FVIIa concentration by comparison to a standard curve constructed with varying concentrations (0.039 to 10 ng/ml) of purified standard FVIIa diluted TBS/BSA.

Western Blotting for FVIIa

To characterize the direct effect of plasmin on FVII activation, FVIIa was assayed in purified systems by Western blotting as follows. FVII (10 μg/ml) and plasmin (0-0.1 U/ml) were incubated at 37°C for 5 min. Reduced samples were run on 10–20% polyacrylamide separating gels according to the method of Laemmli [23]. Following electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Westborough, MA) using a tank electroblotting apparatus in Tris-glycine buffer containing 20% methanol. After the transfer, the membranes were saturated with the blocking buffer to avoid the nonspecific adsorption of proteins, and then incubated with the monoclonal antibody against human FVII/FVIIa. The antibody was then labeled with biotinylated anti(mouse IgG) antibody. Immunostaining was performed after reacting the antibody with biotinylated horseradish peroxidase conjugate using AEC as the substrate. The reaction was terminated after color development by washing with water, and the membrane was dried. The FVIIa generated on the membrane was determined by the comparison of its molecular weight with that of standard FVIIa.

Statistics

Data are means ± standard deviation. Student's paired *t*-test was used to compare the values of two groups. *P* values of <0.05 were considered significant.

RESULTS

The diluted PT was significantly shortened compared with the control by the addition of t-PA (0.1–10 μg/ml)

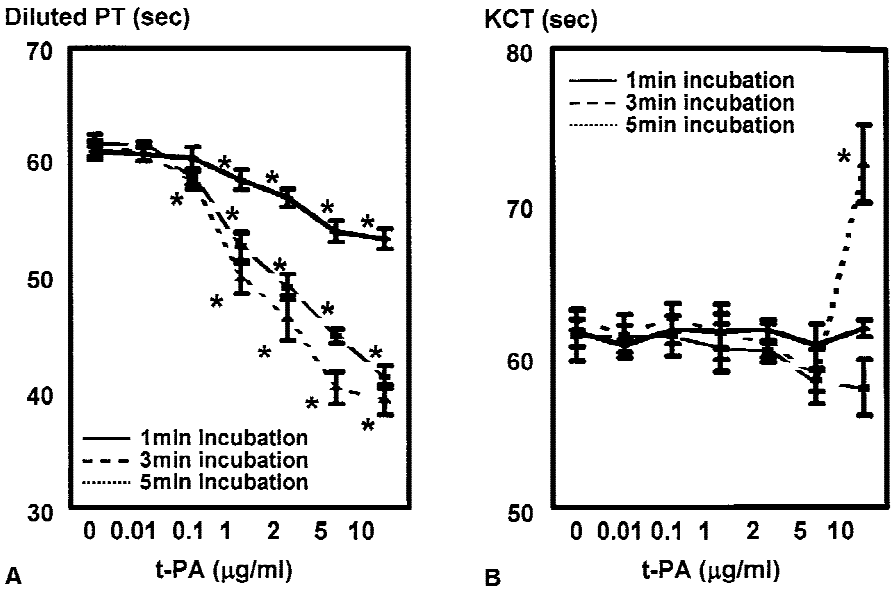

Fig. 1. Effect of t-PA on diluted PT (A) and KCT (B) in NPP. Each value is the mean ± SD of 5 experiments. *, *P* < 0.01 compared with the control.

to NPP (Fig. 1A). This shortening of the diluted PT occurred in an incubation time-dependent (1–5 min) manner. The KCT, however, was not shortened by the addition of t-PA (Fig. 1B). The incubation of NPP with t-PA induced significant concentration-dependent (2–10 μg/ml) increases in the FVIIa levels (Fig. 2). Although FVIIa formation was not observed following the incubation of purified FVII (10 μg/ml) with only t-PA (0–10 μg/ml) (Fig. 3A) or plasminogen (0–10 U/ml) (Fig. 3B), concentration-dependent increases in the FVIIa levels were observed after the incubation of purified FVII (10 μg/ml) with t-PA (0–10 μg/ml) together with plasminogen (1 U/ml) (Fig. 4A), and with only plasmin (0–1 U/ml) (Fig. 4B). To confirm this plasmin-mediated cleavage of FVII to FVIIa, purified FVII (10 μg/ml) was incubated with plasmin (0–0.1 U/ml) for 5 min and the incubation mixture was then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and the Western blotting method. The incubation of FVII with plasmin induced a significant conversion of one-chain FVII to the two-chain form (FVIIa), as demonstrated by the appearance of bands at 50 kd (FVII) and 20 kd (FVIIa) (Fig. 5).

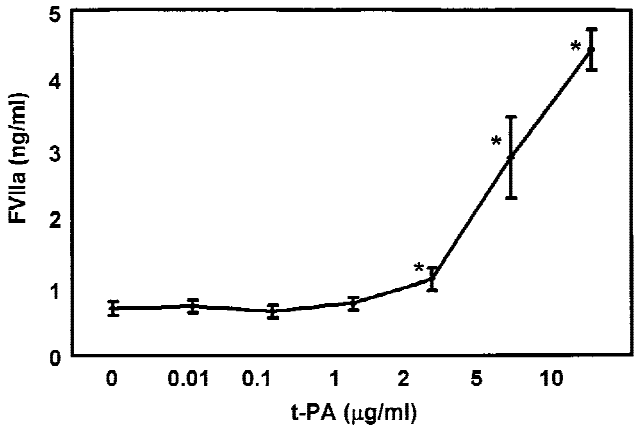

Fig. 2. Effect of t-PA on increases in FVIIa in NPP. Each value is the mean ± SD of 5 experiments. *, *P* < 0.01 compared with the control.

DISCUSSION

To investigate the effect of t-PA on blood coagulation, we examined the effect of t-PA on blood coagulation times in the present study. The diluted PT of NPP was significantly shortened compared with the normal controls by the addition of t-PA at 0.1–10 μg/ml, but the KCT was not shortened. The diluted PT indicates the clotting activity through the coagulation pathway initiated by the conversion of FVII to FVIIa after the addition of ×500-diluted thromboplastin to NPP [20], and the KCT indicates the pathway initiated by the conversion of FXII to FXIIa after the addition of kaolin [21]. Because both of these clotting times of normal plasma are apparently longer than conventional clotting times such as PT and activated partial thromboplastin time (aPTT), the diluted PT and KCT are considered to be suitable to investigate the enhancing activity of coagulation by the shortening of clotting time. Thus, the present findings suggest that t-PA activates the coagulation pathway through FVII activation, but not through FXII activation.

To clarify the activation of FVII by t-PA, we measured the FVIIa levels induced by the addition of t-PA in NPP. The FVIIa levels were dose-dependently increased by the addition of t-PA at 2–10 μg/ml, suggesting that t-PA enhances the activation of FVII. To clarify whether the activation of FVII was the direct effect of t-PA itself or occurred via the generation of plasmin from intrinsic

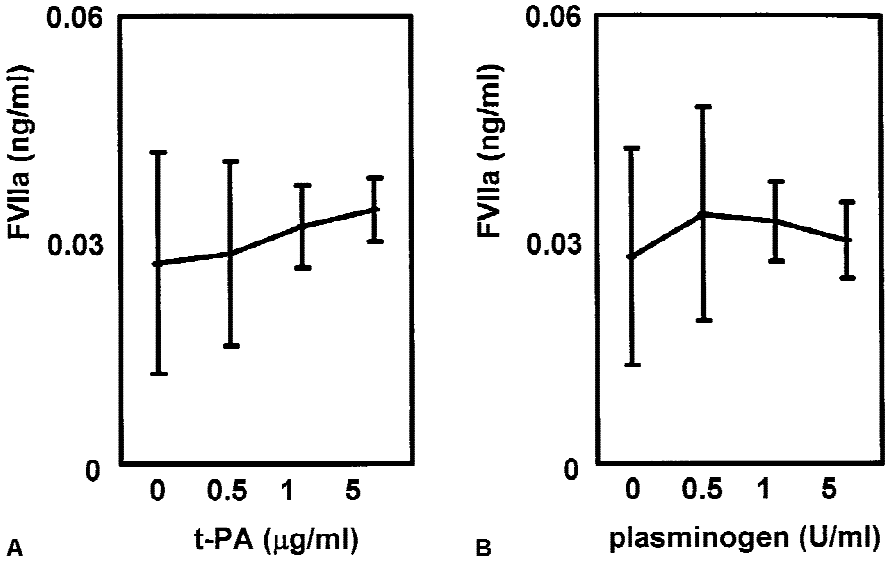

Fig. 3. Effect of t-PA (A) and plasminogen (B) on the activation of purified FVII. Each value is the mean ± SD of 4 experiments.

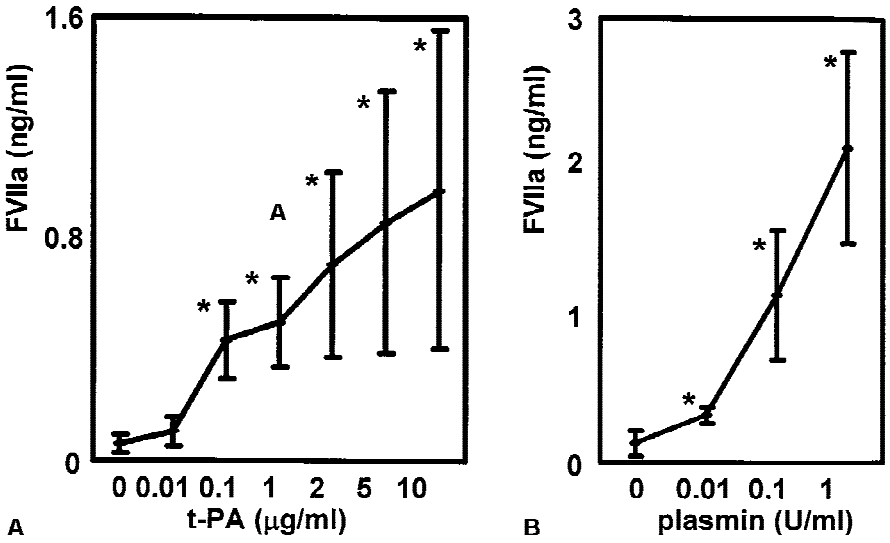

Fig. 4. Effect of t-PA together with plasminogen (1 U/ml) (A) and plasmin (B) on the activation of purified FVII. Each value is the mean ± SD of 5 experiments. *, $P < 0.01$ compared with the control.

plasminogen in NPP, we added t-PA, plasminogen, or plasmin separately to purified FVII solution, and the formation of FVIIa was observed. In purified systems, t-PA together with plasminogen, or plasmin alone has been shown to increase FVIIa when incubated with FVII; however, this FVII formation was not recognized when FVII was incubated with only t-PA or plasminogen in the present study. These findings suggested that plasmin generated from intrinsic plasminogen in NPP by t-PA directly activated FVII. In the kinetic study, the apparent Km value for plasmin-mediated cleavage of FVII was 0.18 μmol/l. This cleavage was similar to those for thrombin- and factor Xa-mediated cleavage of FVII [24].

The enhancing effect of t-PA on diluted PT was observed in an incubation time-dependent manner; i.e., the shortening of diluted PT became more apparent with the increase of incubation time from 1 to 5 min. This result may indicate a stage of the enhancing effect of t-PA. We suspect that the amount of plasmin generated from plasminogen by the addition of t-PA to NPP was increased with the incubation time, and the shortening of diluted PT occurred as a consequence of the increase of FVIIa. The KCT was significantly prolonged after a 5-min incubation by the addition of 10 μg/ml of t-PA. Since the KCT was measured as the time until the clot formation after the addition of kaolin and $CaCl_2$ to NPP, the clot formed might be partly degraded by plasmin generated and accumulated during the long incubation time of NPP with high-dose t-PA. Thus, the KCT of NPP incubated for 5 min with 10 μg/ml of t-PA is thought have been prolonged because of the partial degradation of clot formation by plasmin.

For the activation of FVII, the effect of tissue factor (TF) activity is significant. In the presence of TF, FVIIa activates FX, and FVII is also autoactivated to FVIIa, while FVIIa exhibits very little activity unless it is com-

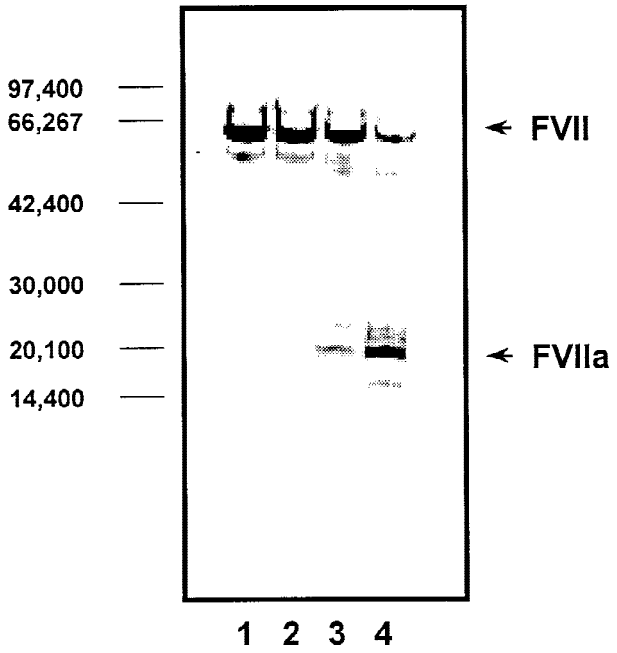

Fig. 5. SDS-PAGE analysis of human plasmin-activated purified human FVII. Lane 1, FVII incubated for 5 min; lane 2, FVII incubated for 5 min with 0.001 U/ml plasmin; lane 3, FVII incubated for 5 min with 0.01 U/ml plasmin; lane 4, FVII incubated for 5 min with 0.1 U/ml plasmin. Numbers at the left indicate the molecular weight.

plexed with TF [25]. A reaction mechanism in which t-PA somehow activated TF activity in NPP and FVIIa was formed effectively may also have been involved in the present results. However, in the purified system without TF, FVIIa was formed from FVII by the addition of plasmin or t-PA plus plasminogen. We measured the TF level in NPP after the addition of t-PA by using an enzyme immunoassay, and found that there was no remarkable effect of t-PA on the TF level in a preliminary study (unpublished results). We also confirmed the direct formation of FVIIa from FVII by plasmin using a Western blotting technique. After the incubation of purified FVII with plasmin for 5 min, the samples were subjected to SDS-PAGE under reducing conditions and the Western blotting method. We observed that the band for FVII at 50 kd had disappeared and the band for FVIIa at 20 kd appeared with the increase of plasmin concentration (0.01 and 0.1 U/ml). These results indicate the direct activation of FVII by plasmin, and this activation system is thus suggested to be unrelated to TF activity.

The mechanism of the activation of the coagulation system by plasmin was reported to depend on the plasmin-mediated activation of FXII [17], FV [26] and FX [27], and the increases of FVII coagulant activity by plasmin [18]. Although these activating mechanisms of the coagulation system are significant, we have observed FVII activation by plasmin that was generated from plasminogen by t-PA in vivo as another important mechanism of the activation of the coagulation system by t-PA. The activation of the coagulation system shown in this study is thought to affect the failure of thrombolytic therapy, at least in part. Bleich et al. [28] reported that heparin therapy after the t-PA therapy of AMI is associated with substantially higher coronary patency rates 3 days after thrombolysis. De Bono et al. [29] reported that concomitant intravenous heparin improves coronary patency in patients with t-PA. These reports suggest that anticoagulation therapy using heparin is useful to preserve the coronary arterial patency after successful thrombolysis with t-PA in AMI patients. Our present results also support the necessity of anticoagulant therapy after thrombolysis.

REFERENCES

1. Loscalzo J, Braunwald E. Tissue plasminogen activator. N Engl J Med 1988;319:925–931.
2. Topol EJ, Morris DC, Swalling RW, Schumacher RR, Taylor CR, Nishikawa A, Liberman HA, Collen D, Tufte ME, Grossberd EB, O'Neill WW. A multicenter, randomized, placebo-controlled trial of a new form of intravenous recombinant tissue-type plasminogen activator (Activase) in acute myocardial infarction. J Am Coll Cardiol 1987; 9:1205–1213.
3. Mueller HS, Rao AK, Forman SA, TIMI iInvestigators. Thrombolysis in myocardial infarction (TIMI): comparative studies of coronary reperfusion and systemic fibrinogenolysis with two forms of recombinant tissue-type plasminogen activator. J Am Coll Cardiol 1987;10: 479–490.
4. Chesebro JH, Knatterud G, Roberts R, Borer J, Cohen LS, Dalen J, Dodge HT, Francis CK, Hillis D, Ludbrook P, Markis JE, Mueller H, Passamani ER, Powers ER, Rao AK, Robertson T, Ross A, Ryan TJ, Sobel BE, Willerson J, Williams DO, Zaret BL, Braunwald E. Thrombolysis in myocardial infarction (TIMI) trial, phase I: a comparison between intravenous tissue plasminogen activator and intravenous streptokinase. Clinical findings through hospital discharge. Circulation 1987;76:142–154.
5. Wilcox RG, von der Lippe G, Olsson CG, Jensen G, Skene AM, Hampton JR, for the asset study group. Trial of tissue plasminogen activator for mortality reduction in acute myocardial infarction. Anglo-Scandinavian study of early thrombolysis (ASSET). Lancet 1988;2: 525–530.
6. ISIS-2 (second international study of infarct survival) collaborative group. Randomised trial of intravenous streptokinase, oral aspirin, both, or neither among 17,187 cases of suspected acute myocardial infarction: ISIS-2. Lancet 1988;2:349–360.
7. Suehiro A, Oura Y, Ueda M, Kakishita E. Inhibitory effect of staphylokinase on platelet aggregation. Thromb Haemost 1993;70:834–837.
8. Suehiro A, Higasa S, Ueda M, Oura Y, Nishida Y, Kakishita E. Effect of plasminogen activator on platelet aggregation. Current aspects of blood coagulation, fibrinolysis and platelets. Shen M-C, Teng C-M, Takada A, editors. Tokyo: Springer; 1993. p 159–164.
9. Owen J, Friedman KD, Grossman BA, Wilkins C, Berke AD, Powers ER. Thrombolytic therapy with tissue plasminogen activator or streptokinase induces transient thrombin activity. Blood 1988;72:616–620.
10. Weitz JI, Cruickshank MK, Thong B, Leslie B, Levine MN, Ginsberg J, Eckhardt T. Human tissue-type plasminogen activator releases fibrinopeptides A and B from fibrinogen. J Clin Invest 1988;82:1700–1707.
11. Rapold HJ, Kuemmerli H, Weiss M, Baur H, Haeberli A. Monitoring of fibrin generation during thrombolytic therapy of acute myocardial

infarction with recombinant tissue-type plasminogen activator. Circulation 1989;79:980–989.
12. Rapold HJ. Promotion of thrombin activity by thrombolytic therapy without simultaneous anticoagulation. Lancet 1990;335:481–482.
13. Eisenberg PR, Miletich JP. Induction of marked thrombin activity by pharmacologic concentrations of plasminogen activators in nonanticoagulated whole blood. Thromb Res 1989;55:635–643.
14. Gulba DC, Barthels M, Westhoff-Bleck M, Jost S, Rafflenbeul W, Daniel WG, Hecker H, Lichtlen PR. Increased thrombin levels during thrombolytic therapy in acute myocardial infarction. Relevance for the success of therapy. Circulation 1991;83:937–944.
15. Munkvad S, Gram J, Jespersen J. Possible role of vascular intima for generation of coagulant activity in patients undergoing coronary thrombolysis with recombinant tissue-type plasminogen activator. A randomized, placebo-controlled study. Scand J Clin Lab Invest 1991; 51:581–590.
16. Eisenberg PR, Sobel BE, Jaffe AS. Activation of prothrombin accompanying thrombolysis with recombinant tissue-type plasminogen activator. J Am Coll Cardiol 1992;19:1065–1069.
17. Ewald GA, Eisenberg PR. Plasmin-mediated activation of contact system in response to pharmacological thrombolysis. Circulation 1995; 91:28–36.
18. Laake K, Osterud B. Activation of purified factor VII by human plasmin, plasma kallikrein, and activation components of the human intrinsic blood coagulation system. Thromb Res 1974;5:759–772.
19. Saito M, Asakura H, Jokaji H, Uotani C, Kumabashiri I, Morishita E, Yamazaki M, Aoshima K, Matsuda T. Mechanism of activation of coagulation induced by tissue-type plasminogen activator. Studies by using coagulation factor deficient plasma. Jpn J Thromb Haemost 1995;6:453–457.
20. Singer JW, Sibley CA. Sensitivity of commercial thromboplastins to factor VII. Am J Clin Pathol 1973;59:755–759.
21. Proctor RR, Rapaport SI. The partial thromboplastin time with kaolin: a simple screening test for first stage plasma clotting factor deficiencies. Am J Clin Pathol 1961;36:212–219.
22. Wildgoose P, Nemerson Y, Hansen LL, Nielsen FE, Glazer S, Hedner U. Measurement of basal levels of FVIIa in hemophilia A and B patients. Blood 1992;80:25–28.
23. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–685.
24. Radcliffe R, Nemerson Y. Activation and control of factor VII by activated factor X and thrombin. J Biol Chem 1975;388–395.
25. Nemerson Y. Tissue factor and hemostasis. Blood 1988;71:1–8.
26. Lee CD, Mann KG. Activation/Inactivation of human factor V by plasmin. Blood 1989;73:185–190.
27. Bebber KH, Zimmermann RE. Procoagulatory effects of plasmin: factor X and prothrombin activation [abstract]. Ann Hematol 1991;1 (Suppl):A42.
28. Bleich SD, Nichols TC, Schumacher RR, Cooke DH, Tate DA, Teichman SL. Effect of heparin on coronary arterial patency after thrombolysis with tissue plasminogen activator in acute myocardial infarction. Am J Cardiol 1990;66:1412–1417.
29. De Bono DP, Simoons ML, Tijssen J, Arnold AER, Betriu A, Burgersdijk C, Bescos LL, Mueller E, Pfisterer M, Van de Werf F, Zijlstra F, Verstraete M, for the European Cooperative Study Group. Effect of early intravenous heparin on coronary patency, infarct size, and bleeding complications after alteplase thrombolysis: results of a randomised double blind European Cooperative Study Group trial. Br Heart J 1992;67:122–128.